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(54) A microorganism for selective production of a specific component of avermectin and a method for selective production thereof.

(57)

1. A microorganism of the genus Streptomyces which is capable of selectively producing a specific component of avermectin, said microorganism having one or more of the following properties:

- specific accumulation of avermectin component "a",
- effective incorporation of isoleucine or the keto acid thereof (3-methyl-2-oxovaleric acid) into an avermectin molecule, and
- markedly suppressed incorporation of valine or the keto acid thereof (2-oxoisovaleric acid) into an avermectin molecule.

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Field of the Invention

This invention relates to a microorganism belonging to genus Streptomyces for selective production of a specific component of avermectin having the properties of specific accumulation of avermectin component "a", an effective incorporation of isoleucine or its keto acid (3-methyl-2-oxovaleric acid) into avermectin structure, and markedly suppressed incorporation of valine or its keto acid (2-oxoisovaleric acid) into avermectin structure. More particularly the present invention concerns a process for selective production of a specific component of avermectin using microorganism belonging to genus Streptomyces which is in defect with avermectin B O-methyltransferase activity.

The Prior Arts

Avermectins are an antibiotic having anthelmintic activity produced by Streptomyces avermitilis. In cultured medium of the said microorganism, eight components of avermectin, A_{1a} , A_{2a} , B_{1a} , B_{2a} , A_{1b} , A_{2b} , B_{1b} and B_{2b} , are produced (U.S. Patent, 4310,519). Components "A" and "B" have substituent methoxy or hydroxy at C - 5, respectively. A component group "1" has double bond at C - 22 and C - 23, and group "2" has hydrogen at C - 22 and hydroxy at C - 23. An "a" group component has substituent sec-butyl at C - 25, and a "b" group component has substituent isopropyl at C - 25. Among these 22, 23-dihydroavermectin B_1 - (Ivermectin), a hydrogenated product of B_1 component, is used as an anthelmintic.

In the prior art, avermectin has been produced by culturing Streptomyces avermitilis in a medium consisting of an assimirable nitrogen source, carbon source and inorganic salt under aerobic condition to produce analogous structure eight components of avermectin A_{1a} , A_{2a} , B_{1a} , B_{2a} , A_{1b} , A_{2b} , B_{1b} and B_{2b} . Extracted product with organic solvent, a mixture of avermectins, are separated to a fraction of A_1, A_2, B_1 and B_2, then purified to obtain B_1 fraction, which is a mixture of B_{1a} and B_{1b} , thereafter the B_1 fraction is hydrogenated to manufacture 22 23-dihydroavermectin B_1.

The prior art has number of disadvantages. Namely, the eight components have to be produced as a mixture. Furthermore an industrial scale separation of "a" and "b" components are quite difficult, thereby an efficient production of B_{1a} component with good yield and low cost has strongly been requested.

Summary of the Invention

We have found that avermectin B_{1a} and B_{2a} components can effectively be obtained by using a microorganism strain having the properties of specific accumulation of avermectin component "a", an effective incorporation of isoleucine or its keto acid (3-methyl-2-oxovaleric acid) into avermectin structure, and markedly suppressed incorporation of valine or its keto acid (2-oxoisovaleric acid) into avermectin structure, to which adding a deficiency of avermectin B O-methyltransferase activity. Separation of avermectin B_{1a} (a double bond between C - 22 and C - 23) and B_{2a} (OH at C - 23) components can easily be made by chromatography.

Objects of the Invention

An object of the present invention is to provide a process for selective production of a specific component of avermectin which comprises culturing a microorganism belonging to Streptomyces and having the properties of specific accumulation of avermectin component "a", effective incorporation of isoleucine or its keto acid (3-methyl-2-oxovaleric acid) into avermectin structure, and markedly suppressed incorporation of valine or its keto acid (2-oxoisovaleric acid), to which adding a deficiency of avermectin B O-methyltransferase activity, in a medium, accumulating avermectin B_{1a} and B_{2a} in a medium, and isolating avermectin B_{1a} and B_{2a} from the cultured mass.

Another object of the present invention is to provide a microorganism belonging to Streptomyces avermitilis for selective production of specific component of avermectin having the properties of specific accumulation of avermectin component "a", an effective incorporation of isoleucine or its keto acid (3-methyl-2-oxovaleric acid) into avermectin structure, and markedly suppressed incorporation of valine or its keto acid (2-oxoisovaleric acid) in to avermectin structure, to which adding a deficiency of avermectin B O-methyltransferase activity.

Brief Description of the Drawings

Figure 1A shows the HPLC pattern of a cultured material of Streptomyces avermitilis ATCC 31271 ;

Figure 1B shows the HPLC pattern of a cultured material of Streptomyces avermitilis K 2033 ;

Figure 2 shows the HPLC pattern of cultured material of Streptomyces avermitilis K 2038 ;
Figure 3 shows the HPLC pattern purified product of cultured Streptomyces avermitilis K 2038.

Detailed Description of the Invention

No report has been known on the effective accumulation of specific component of avermectin by a fermentation method without feeding additives in a medium during culture using microorganism belonging to Streptomyces avermitilis to which the deficient nature is incorporated.

The microorganisms having the properties of specific accumulation of avermectin component "a", an effective incorporation of isoleucine or its keto acid (3-methyl-2-oxovaleric acid) into avermectin structure, and markedly suppressed incorporation of valine or its keto acid (2-oxoisovaleric acid) into avermectin structure, to which adding a deficiency of avermectin B O-methyltransferase activity, can be used in the present invention whether it is obtained from natural sources or mutant having auxotrophic nature or drug resistance, and can be included in the present invention. The present invention also includes microorganisms, a mutant having the properties described in the present specification, which are improved by recombinant DNA techniques, transformation or transduction.

The preferable example of microorganism used in the present invention is Streptomyces avermitilis K 2038 which is derived from Streptomyces avermitilis ATCC 31271, and is a mutant wherein protoplasts of a mutant K 2033 having the properties of specific accumulation of avermectin component "a", an effective incorporation of isoleucine or its keto acid (3-methyl-2-oxovaleric acid) into avermectin structure, and markedly suppressed incorporation of valine or its keto acid (2-oxo-isovaleric acid) into avermectin structure, and a mutant K 2034 having a deficient nature of avermectin B O-methyl-transferase activity, are fused. Namely strain K 2038 is a mutant in which a deficient nature of avermectin B O-methyl-transferase activity is introduced into a strain K 2033. These mutant strains, Streptomyces avermitilis K 2033, K 2034 and K 2038, have been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, M.I.T.I. Japan, according to Budapest Treaty as FERM BP-2773, FERM BP-2774 and FERM BP-2775, respectively.

Induction in mutation can easily be performed by conventional mutation techniques. Preferably, original strain is treated by ultraviolet irradiation or with a mutagen such as N-methyl-N'-nitro-N-nitrosoguanidine or ethyl methanesulfonic acid, thereafter treated colonies are cultured in a medium, in which labeled isoleucine or its keto acid (3-methyl-2-oxo-valeric acid) and labeled valine or its keto acid (2-oxo-isovaleric acid) are added and the culture is further continued for several hours. Avermectin fraction is isolated from cultured mycelia and radioactivity of avermectin is measured, thereby mutants having properties of an effective incorporation of isoleucine or its keto acid (3-methyl-2-oxovaleric acid) into avermectin structure, and significantly suppressed incorporation of valine or its keto acid (2-oxo-isovaleric acid) into avermectin sturcture, are selected.

A mutant deficient in avermectin B O-transferase activity can be obtained by treatment of mutagen as same as of the above, and the thus obtained colonies are cultured in avermectin production medium. Cultured mycelia are extracted with organic solvent and the extract is separated by silica-gel thin layer chromatography. Then the mutant which can merely produce avermectin B can be selected.

An introduction of the deficiency of avermectin B O-methyltransferase activity by a protoplast fusion technique can be performed by that a protoplast prepared from a mutant having the properties of specific accumulation of avermectin component "a", an effective incorporation of isoleucine or its keto acid (3-methyl-2-oxovaleric acid) into avermectin structure, and markedly suppressed incorporation of valine or its keto acid (2-oxo-isovaleric acid) into avermectin structure, and a protoplast prepared from a mutant having the properties of deficient in avermectin B O-methyltransferase activity, are fused with using polyethylene glycol, then the fused protoplast is regenerated to mycelia in a suitable regeneration medium. Thus regenerated colonies having the properties of accumulating avermectin component "a" and deficient in avermectin B O-methyl-transferase activity are selected.

In a production of avermectin B_{1a} and B_{2a} , the mutant strain having the properties of specific accumulation of avermectin component "a", an effective incorporation of isoleucine or its keto acid (3-methyl-2-oxovaleric acid) into avermectin structure, and markedly suppressed incorporation of valine or its keto acid (2-oxoisovaleric acid) into avermectin structure, and having a deficient nature of avermectin B O-methyltransferase activity, are cultured in a medium. A medium for production of avermectin B_{1a} and B_{2a} is a conventional medium which contains carbon source, nitrogen source and inorganic salts. Examples of carbon source are glucose, glycerin, sucrose, dextrin, starch or molasses. Examples of nitrogen source are caseine, caseine hydrolysate, yeast extract, autolysed yeast, yeast hydrolysate, dry yeast, soy bean powder, soy bean digestible, corn steep liquor, distillar's soluble, cotton seed powder or meat extract. Examples of inorganic salts are phosphate, sulfate, nitrate, chloride or carbonate of sodium, potassium,

magnesium, ammonium, calcium, manganese, zinc, iron or cobalt, and a conventional salt which can generate these ions. Cultivation can be proceeded under aerobic condition. Fermentation can be proceeded by controlling the medium at pH 5 - 9, at 25 - 35 °C, for 120 - 192 hours shaking culture under aerobic condition. If the production of avermectin B_{1a} is desired, an avermectin producing strain which can accumulate avermectin B_{1a} should naturally be selected.

Avermectin B_{1a} and B_{2a} can be isolated from cultured mass by a conventional isolation method for antibiotics. For example, a composition containing avermectins is extracted the filtered mycelia with organic solvent such as acetone or methanol, which is concentrated after filtration. The concentrate is further extracted with organic solvent such as methylenechloride. Organic layer was concentrated in vacuo to obtain avermectin B_{1a} and B_{2a}. Avermectin B_{1a} can be separated by treating the concentrate with column chromatography using ion-exchanger, silica-gel, reverse phase silica-gel or Sephadex, or counter current distribution method. For example, a mixture containing avermectin B_{1a} is treated by preparative HPLC (reverse phase silica-gel, ODS) with migration phase of 80% v/v methanol/water to elute avermectin B_{1a} . The extract is concentrated in vacuo and recrystallized from methanol to obtain avermectin B_{1a} in a pure form.

Following examples illustrate the present invention but are not construed as limiting.

Isolation of a mutant having the properties of specific accumulation of avermectin component "a", an effective incorporation of isoleucine or its keto acid (3-methyl-2-oxovaleric acid) in to avermectin structure, and markedly suppressed incorporation of valine or its keto acid (2-oxoisovaleric acid) into avermectin structure:

Example 1

Spores of Streptomyces avermitilis ATCC 31271 treated with conventional method by N-methyl-N'-nitro-N-nitrosoguanidine (1 mg/ml, pH 9.0 at 30 °C for 60 min.) treatment were diluted with sterilized water for approximately 200 colonies per plate, spread on YMS plate and cultured at 30 °C for 5 days. The colonies were picked up and inoculated patchily onto YMS plate with 1 cm^2 square, for which was set up as a master plate. Each colony on the master plate was inoculated into a 100 ml Erlenmeyer flask containing 10 ml of culture medium, and cultured at 28 °C with 210 rpm, amplitude 2.5 cm for 96 hours. [U-^{14}C] -L-isoleucine (50,000 cpm) and [3,4-^{3}H] -L-valine (100,000 cpm) were added thereto and further cultured for 6 hours. Cultured mycelia were collected and extracted with 3 ml acetone. The acetone extract was dried in vacuo. Crude extract dissolved in a small amount of methanol was spotted on a silica-gel thin layer plate (Merck Kiesel gel 60F$_{254}$) and developed with 15% v/v isopropanol/hexane. After checking avermectin spot by UV irradiation at 254 nm, the part thereof was cut and put into 15 ml scintillation vial, added 0.5 ml methanol, and shaked at room temperature for 10 minutes to extract avermectin from silica-gel. 5 ml scintillator (10 g 2,5 -diphenyloxazol, 0.2 g p-bis(O-methylstyryl) benzune and 1 ℓ xylene) was added therein and radioactivity of each sample was measured by liquid scintillation spectrometer. Streptomyces avermitilis ATCC 31271 was used as a control.

Example 2

A corresponding mutant having the properties of an effective incorporation of [U-^{14}C] -L-isoleucine into avermectin structure, and markedly suppressed incorporation of [3,4-^{3}H] -L-valine into avermectin structure, was collected from the master plate and cultured as same as of in Example 1. An equal amount as of in Example 1 of [U-^{14}C] -L-isoleucine and [3,4-^{3}H] -L-valine, or [U-^{14}C] -3-methyl-2-oxovaleric acid and [3,4-^{3}H] -2-oxoisovaleric acid were added and cultured for 6 hours. Avermectin fraction was isolated as same as of in Example 1 and the radio activity was measured. Result is shown in Table 1. Labeled compound was incorporated into avermectin structure by an original strain Streptomyces avermitilis ATCC 31271. On the other hand, a mutant strain K 2033 incorporated [U-^{14}C] - L-isoleucine or its keto acid (3-methyl-2-oxovaleric acid) effectively into avermectin structure, but incorporation of [3,4-^{3}H] -L-valine or its keto acid (2-oxoisovaleric acid) into avermectin structure was markedly decreased.

Example 3

Spore suspensions of the original strain Streptomyces avermitilis ATCC 31271 and strain K 2033 obtained in Example 2 were inoculated into 100 ml Erlenmeyer flask containing 10 ml production medium, and cultured at 28 °C, 210 rpm, amplitude 2.5 cm. for 168 hours. Cultured mycelia was mixed with 10 ml

methanol and extracted the avermectin. After removing mycelial residue by centrifugation, avermectin in the extract was analysed by HPLC (ODS 3 μm, column size 6 ø × 75 mm, speed 1 ml/min., mobility phase 80% v/v methanol/water, detection 246 nm). Result is shown in Fig. 1A and 1B. An original strain Streptomyces avermitilis ATCC 31271 produced 8 components of avermectin (A_{1a} , A_{1b} , A_{2a} , A_{2b} , B_{1a} , B_{1b} , B_{2a} and B_{2b}) and the mutant strain K 2033 produced 4 components of avermectin containing main effective component B_{1a} (A_{1a} , A_{2a} , B_{1a} and B_{2a}).

Mutant strain defect in avermectin B O-methyltransferase

Example 4

Spores of Streptomyces avermitilis ATCC 31271 treated with conventional method by N-methyl-N'-nitro-N-nitrosoguanidine (1 mg/ml, pH 9.0 at 30 °C for 60 min.) treatment were diluted with sterilized water for approximately 200 colonies per plate, spread on YMS plate and cultured at 30 °C for 5 days. The colonies were picked up and inoculated patchily onto YMS plate with 1 cm^2 square, for which was set up as a master plate. The master plate was replicated on the production medium 3 and incubated at 30°C for 8 days. Each patch-like shape colony on the production medium 3 was cut-out together with agar medium, inserted into plastic tube, added 0.5 ml acetone therein and allowed to stand at room temperature for 15 minutes to extract the cultured product. After removing mycelia and agar strips, the acetone extract was dried in vacuo. Extract was dissolved in 25μl acetone, and 5μl thereof were spotted on a silica-gel thin layer plate (Merck, Kiesel gel $60F_{254}$) then developed with 15% v/v isopropanol/hexane. A mutant strain K 2034 which produced only avermectin B component was collected by means of UV irradiation at 254 nm.

Introduction of avermectin B O-methyltransferase deficient property into a strain K 2033 by protoplast fusion:

Example 5

A spore suspension of strain K 2033 obtained in Example 2 and avermectin B O-methyltransferase activity deficient strain K 2034 obtained by mutation of orinal strain ATCC 31271 was inoculated into 500 ml Erlenmeyer flask containing YEME medium (50 ml) including 30% w/v sucrose, 5mM $MgCl_2$ and 0.5% w/v glycine, and cultured at 28°C , 180 rpm for 68 hours. Mycelia obtained by centrifugation of cultured liquid at 3000 rpm for 10 min. was suspended in P10 medium (10 ml) and re-centrifuged to collect myceria. Washed mycelia were suspended in P10 medium (10 ml) containing egg white lysozyme (1 mg/ml) sterilized by filtration and gently shaked at 37°C for 60 minutes to form protoplast. A sample containing the protoplast was filtered through cotton filter to remove undigested mycelia. Protoplast was sedimented by centrifugation at 3000 rpm for 10 minutes. Protoplast was gently suspended by adding P 20 medium (5 ml) thereafter again protoplast was collected by centrifugation. After resuspended the protoplastin P 20 medium (2 ml), a part of the suspendion was diluted and dropped onto hemocytometer then number of protoplast was calcurated by phase-contrast microscope (× 400). The protoplasts of a strain K 2033 and K 2034, each 5 × 10^8, respectively, were transferred into small test tube and mixed completely. Whole volume thereof was controlled below 50 μl. A solution (0.5 ml) of 50% w/v polyethylene glycol #1000 (1g of polyethylene glycol #1000 dissolved in 1ml of P 20 medium was filter-sterilized through 0.45 μm filter) was added therein and rapidly mixed to fuse the protoplasts. After allowed to stand at room temperature for 1 minutes, P 20 medium (0.5 ml) was added and mixed to dilute polyethylene glycol. A fusant was diluted with P 20 medium at 10 $^{-2}$ and 10 $^{-3}$, and 0.1 ml/plate of the fusant and 2.5 ml of soft agar RM14 were spread on RM14 medium. The plate was incubated at 30 °C for 10 days to regenerate mycelia. Mycelia was separated from the plate surface and homogenated by homogenizer. The mycelia was diluted with sterile water, spread on YMS plate and incubated at 30°C for 5 days. Matured spore was scratched up, diluted with sterile water up to 200 colonies per plate, spread on YMS plate then incubated at 30°C for 5 days. 80% of the budding colonies showed type of K 2033 strain (dark brown, abundant spores) and the other showed type of K 2034 strain (pale brown, few spores).

Example 6

Colonies appearing analogous type of K 2033 strain obtained by protoplast fusion were spread patchily and incubated at 30 °C for 8 days. Patches of each colony were cut-out, inserted into plastic tubes, added acetone (0.5 ml) thereto and allowed to stand at room temperature for 15 minutes for extracting the product.

Mycelia and agar strips were removed off and the extract was concentrated in vacuo. The thus obtained crude extract was dissolved in acetone (25 μl) and the solution (5 μl) was spotted on a silica-gel thin layer plate, then developed with 85% v/v hexane/isopropanol. Avermectin thus produced was checked by UV at 254 nm, then strains which produced avermectin B component having deficient nature of avermectin B O-methyltransferase activity were selected. Further these extracts were spotted on a reversed phase silica-gel thin layer plate (Whatman KC18F), developed with 70% v/v acetonitrile/water, then strains merely producing avermectin "a" component were selected by checking with UV at 254 nm.

Example 7

Spore suspension of a variant (K 2038 strain) which was obtained by protoplast fusion and was merely producing avermectin "a" component in B component, was inoculated in 50 ml large test tube containing 10 ml seed medium and shake cultured at 30 °C for 48 hours. A 0.2 ml thereof was inoculated into 100 ml Erlenmeyer flask containing 10 ml production medium 2 and cultured at 28°C at 210 rpm, 2.5 cm amplitute for 168 hours. Cultured product was extracted by the same way as of in Example 3, then the extract was analysed. Result is shown in Fig. 2. A strain K 2038 obtained by protoplast fusion produced the active principles components B_{1a} and B_{2a} in the 8 components of avermectins. Furthermore amount of accumulation of avermectin B_{1a} wassignificantly increased up.

Example 8

Mycelia was separated from cultured mass (approx. 250 ml) of Streptomyces avermitilis K 2038 cultured in 30 Erlenmeyere flasks. After washing the mycelia with deionized water (150 ml), methanol (100 ml) was added thereto and stirred at room temperature for 30 minutes for extraction. Mycelial extract was filtered through Celite to remove mycelial residue, and the filtrate was concentrated up to approximately 10 ml in vacuo. Deionized water (10 ml) further methylenechloride (20 ml) were added to the concentrate for extraction. Methylenechloride layer was separated, thereafter futher the water layer was extracted with methylenechloride (20 ml). Combined methylenechloride extract was dried up in vacuo and the residue was dissolved in ethyl acetate (50 ml) which was dehydratd by adding anhydrous sodium sulfate (2 g). Ethyl acetate solution was passed through silica-gel (10 g) column then eluted with ethyl acetate (50 ml). Eluate was collected and concentrated in vacuo to obtain viscous oily product (0.8 g). The oily substance (0.8 g) dissolved in small amount of methylenechloride was passed through a column of silica-gel (Merck 100 - 200 mesh) equilibrated with methylenechloride. After washing with methylenechloride, elution was carried out with 5% v/v isopropanol/methylenechloride. Fractions containing avermectin B_{1a} was collected then passed through a column of active carbon (2 g). The column was further washed with methylenechloride (10 ml). The thus obtained eluate was dried up in vacuo, added thereto small amount of isopropyl ether to dissolve the material, thereafter hexane was added dropwise under cooling to obtain white precipitate. The precipitate was collected by filtration, dried in vacuo to obtain white powder (10 mg). According to analysis by HPLC the said white powder contained over 90% of avermectin B_{1a}. (refer to Fig. 3)

Physico-chemical properties of the thus obtained white powder is identical with those of avermectin B_{1a} reported in J. Am. Chem. Soc., 103: 4216 - 4221 (1981).

Compositions of the media used in the foregoing examples are illustrated hereinbelow.

YMS medium

malt extract	(Difco)	10 g
yeast extract	(Difco)	4 g
soluble starch	(Difco)	4 g
agar		20 g
distilled water		1 ℓ

Adjusted to pH 7.2 with adding 2N KOH and sterilized at 121 °C for 15 minutes. After sterilization, magnesium chloride and calcium nitrate were added up to 10 mM and 8 mM, respectively.

Production medium 1

glucose	30 g
NaCℓ	2.0 g
KH_2PO_4	0.05 g
$FeSO_4 \cdot 7H_2O$	0.05 g
$ZnSO_4 \cdot 7H_2O$	0.05 g
$MnSO_4 \cdot 4H_2O$	0.05 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$(NH_4)_2SO_4$	1.5 g
$CaCO_3$	5.0 g
distilled water	1 ℓ

Adjusted to pH 7.2 with 2N KOH, then sterilized at 121 °C for 15 min.

Production medium 2

glucose	45 g
peptonized milk (Oxoid)	24 g
autolyzed yeast (Difco)	2.5 g
polypropyleneglycol #2000	2.5 mℓ
distilled water	1 ℓ

Ajdusted to pH 7.2 with 2N KOH, then sterilized at 121 °C for 15 minutes.

Production medium 3

glucose	45 g
peptonized milk (Oxoid)	24 g
autolyzed yeast (Difco)	2.5 g
agar	20 g
distilled water	1 ℓ

Adjusted to pH 7.2 with 2N KOH, then sterilized at 121 °C for 15 minutes.

YEME medium

yeast extract	(Difco)	3 g
malt extract	(Difco)	3 g
peptone	(Difco)	5 g
glucose		10 g
distilled water		1 ℓ

Sterilized at 121 °C for 15 minutes.

Trace element solution

$FeC\ell_3 \cdot 6H_2O$	200mg
$ZnC\ell_2$	40mga
$CuC\ell_2 \cdot 2H_2O$	10mg
$MnC\ell_2 \cdot 4H_2O$	10mg
$Na_2B_4O_7 \cdot 10H_2O$	10mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	10mg
distilled water	1 ℓ

P 10 medium

sucrose	103 g
K_2SO_4	0.25 g
$MgC\ell_2 \cdot 6H_2O$	2.03 g
trace element solution	2.0 mℓ
distilled water	800 mℓ

After sterilization at 121 °C for 15 minutes following mixture is added.

0.5 % KH_2PO_4	10 mℓ
3.68 % $CaC\ell_2 \cdot 2H_2O$	100 mℓ
0.25 M MES buffer solution pH 6.5	100 mℓ

P 20 medium

sucrose	205 g
K_2SO_4	0.25 g
$MgC\ell_2 \cdot 6H_2O$	2.03 g
trace element solution	2.0 mℓ
distilled water	800 mℓ

After sterilization at 121 °C for 15 minutes following mixture is added.

0.5 % KH_2PO_4	10 mℓ
3.68 % $CaC\ell_2 \cdot 2H_2O$	100 mℓ
0.25 M MES buffer solution pH 6.5	100 mℓ

RM 14 medium

sucrose	205 g
K_2SO_4	0.25 g
$MgC\ell_2 \cdot 6H_2O$	10.12 g
glucose	10.0 g
casamino acid	0.1 g
L-proline	3.0 g
yeast extract (Difco)	2.0 g
trace element solution	2.0 mℓ
oat meal agar (Difco)	3.0 g
agar	20 g
distilled water	870 mℓ

After sterilized at 121 °C for 15 minutes follwoing mixture is added.

0.05 % KH_2SO_4	10 mℓ
3.68 % $CaC\ell_2 \cdot H_2SO_4$	80 mℓ
0.25 M MES buffer solution pH 6.5	40 mℓ

Soft agar RM 14 medium

sucrose	205 g
K_2SO_4	0.25 g
$MgCl_2 \cdot 6H_2O$	10.12 g
glucose	10.0 g
casamino acid (Difco)	0.1 g
L-proline	3.0 g
yeast extract (Difco)	2.0 g
trace element solution	2.0 mℓ
oat meal agar (Difco)	3.0 g
agar	5.0 g
distilled water	870 mℓ

After sterilized at 121 °C for 15 minutes following mixture is added.

0.5 % KH_2PO_4	10 mℓ
3.68 % $CaCl_2 \cdot 6H_2O$	80 mℓ
0.25 M MES buffer solution pH 6.5	40 mℓ

Table 1

strain	Incorporation of labeled compound into avermectin (cpm)			
	Additive			
	(U-^{14}C)-L-isoleucine	(3, 4-^{3}H)-L-valine	(U-^{14}C)-3-methyl-2-oxovaleric acid	(3, 4-^{3}H)-2-oxovaleric acid
ATCC31271	398	423	655	575
K2033	512	22	927	25

Claims

1. A microorgnism of the genus Streptomyces which is capable of selectively producing a specific component of avermectin, said microorganism having one or more of the following properties:

- specific accumulation of avermectin component "a",
- effective incorporation of isoleucine or the keto acid thereof (3-methyl-2-oxovaleric acid) into an avermectin molecule, and
- markedly suppressed incorporation of valine or the keto acid thereof (2-oxoisovaleric acid) into an avermectin molecule.

2. A microorganism according to claim 1 which is Streptomyces avermitilis.

3. A microorganism according to claim 2 which is a mutant of Streptomyces avermitilis ATCC 31271.

4. A microorganism according to claim 3 which is Streptomyces avermitilis K 2033 FERM BP-2773 or a mutant thereof.

5. A microorganism according to claim 3 which is Streptomyces avermitilis K 2034 FERM BP-2774 or a mutant thereof.

6. A microorganism according to claim 3 which is Streptomyces avermitilis K 2038 FERM BP-2775 or a mutant thereof.

7. A microorganism according to any one of the preceding claims which is deficient in avermectin B O-methyltransferase activity.

8. A process for preparing a microorganism as defined in any one of the preceding claims which comprises mutating a microorganism of the genus Streptomyces and selecting microorganisms having one or more of the properties defined in claim 1.

9. A process for selective production of a specific component of avermectin which comprises culturing a microorganism as defined in any one of claims 1 to 8, allowing avermectin B_{1a} to accumulate in the cultured mass, and isolating avermectin B_{1a} therefrom.

10. A process for selective production of a specific component of avermectin which comprises culturing a microorganism as defined in any one of claims 1 to 8, allowing avermectin B_{1a} and B_{2a} to accumulate in the cultured mass, and isolating avermectin B_{1a} and B_{2a} therefrom.

Claims for the following Contracting State: ES

1. A process for preparing a microorganism of the genus Streptomyces which is capable of selectively producing a specific component of avermectin, said microorganism having one or more of the following properties:
   - specific accumulation of avermectin component "a",
   - effective incorporation of isoleucine or the keto acid thereof (3-methyl-2-oxovaleric acid) into an avermectin molecule, and
   - markedly suppressed incorporation of valine or the keto acid thereof (2-oxoisovaleric acid) into an avermectin molecule;

   said proecess comprising mutating a microorganism of the genus Streptomyces and selecting microorganisms having one or more of the properties defined above.

2. A proecess according to claim 1 wherein the starting microorganism is Streptomyces avermitilis.

3. A process according to claim 2 wherein the starting microorganism is Streptomyces avermitilis ATCC 31271.

4. A process according to claim 3 wherein the microorganism obtained is Streptomyces avermitilis K 2033 FERM BP-2773.

5. A process according to claim 3 wherein the microorganism obtained is Streptomyces avermitilis K 2034 FERM BP-2774.

6. A process according to claim 3 wherein the microorganism obtained is Streptomyces avermitilis K 2038 FERM BP-2775.

7. A process according to any one of the preceding claims wherein the microorganism obtained is deficient in avermectin B O-methyltransferase activity.

8. A process for selective production of a specific component of avermectin which comprises culturing a microorganism of the genus Streptomyces as defined in any one of the preceding claims, said microorganism having one or more of the properties defined in claim 1, allowing avermectin B_{1a}, or B_{1a} and B_{2a}, to accumulate in the cultured mass, and isolating avermectin B_{1a}, or B_{1a} and B_{2a}, therefrom.

FIG. 1A

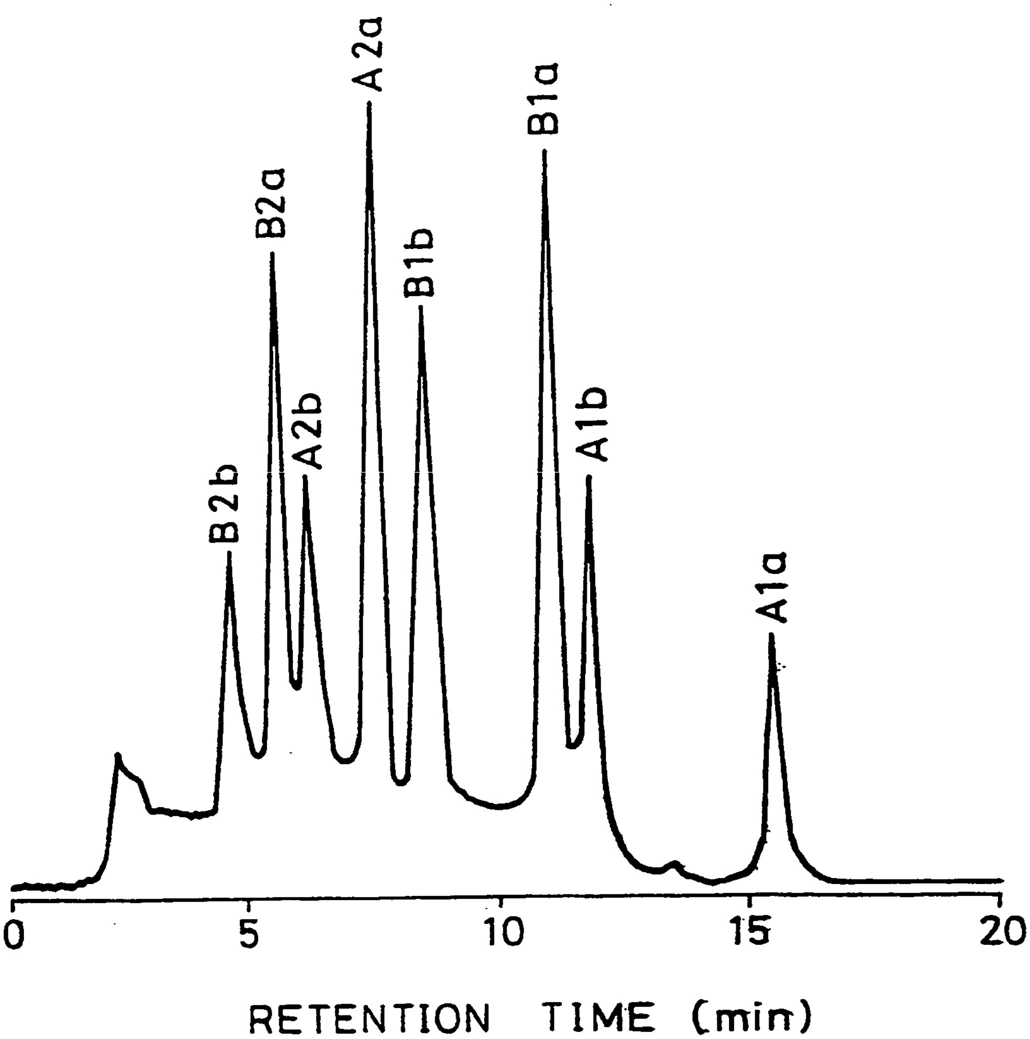


FIG. 1B

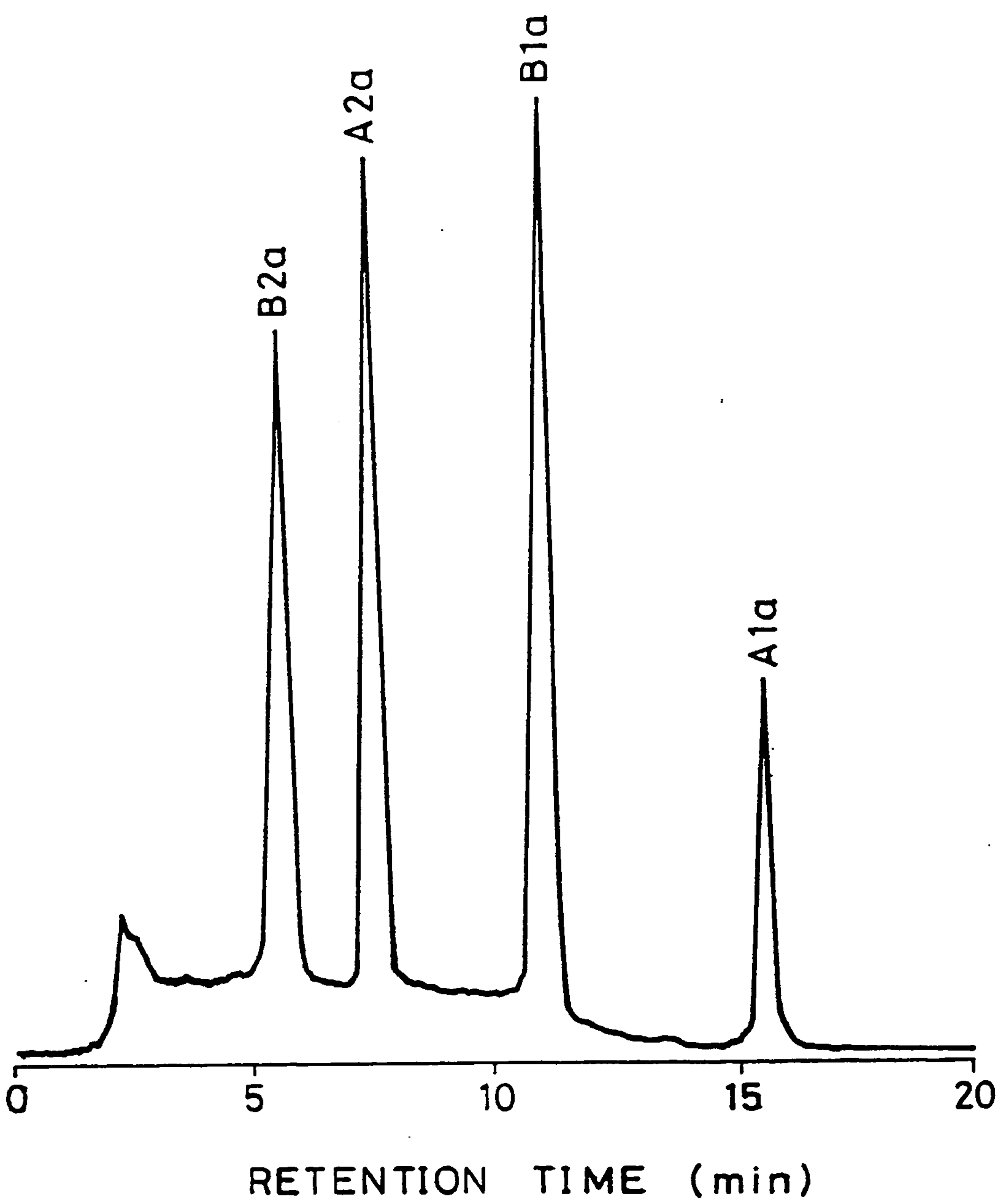


FIG. 2

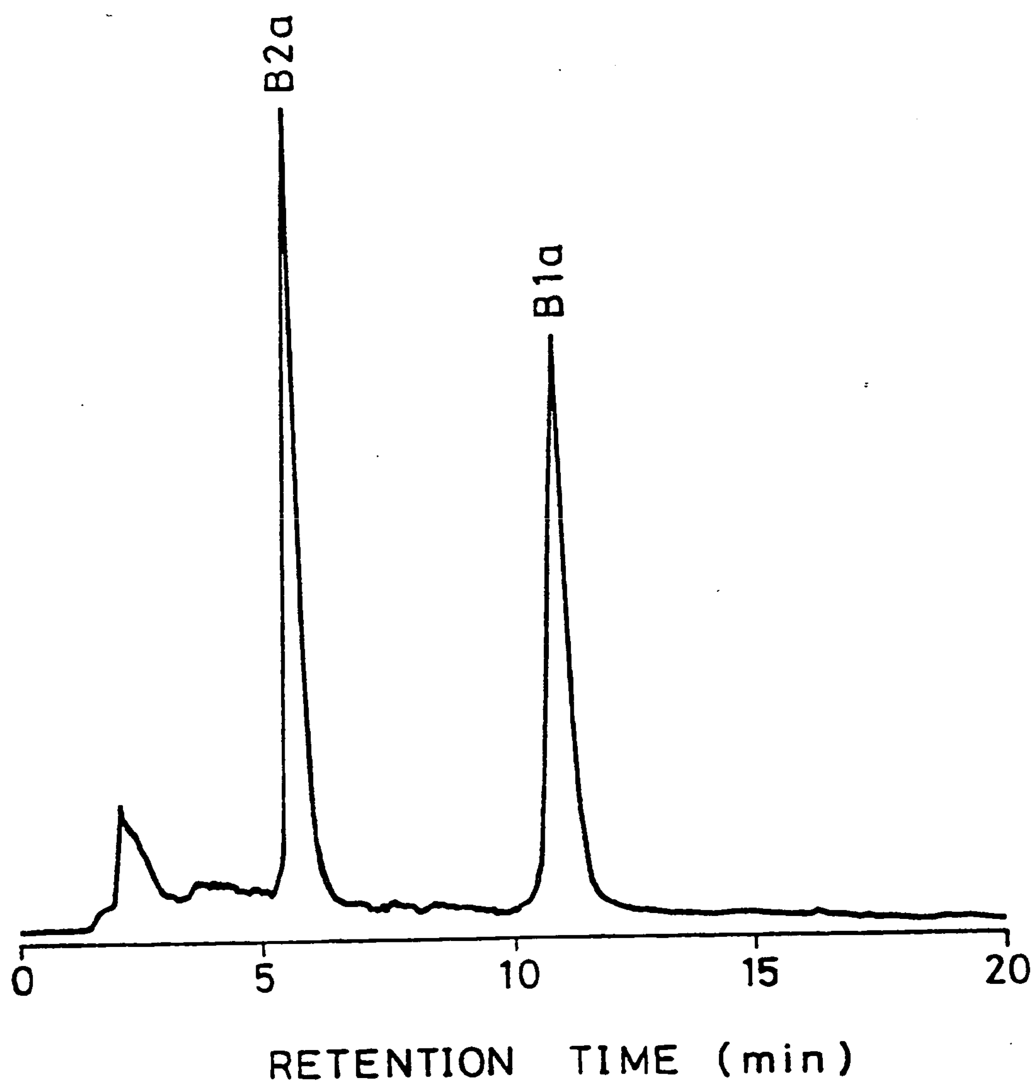
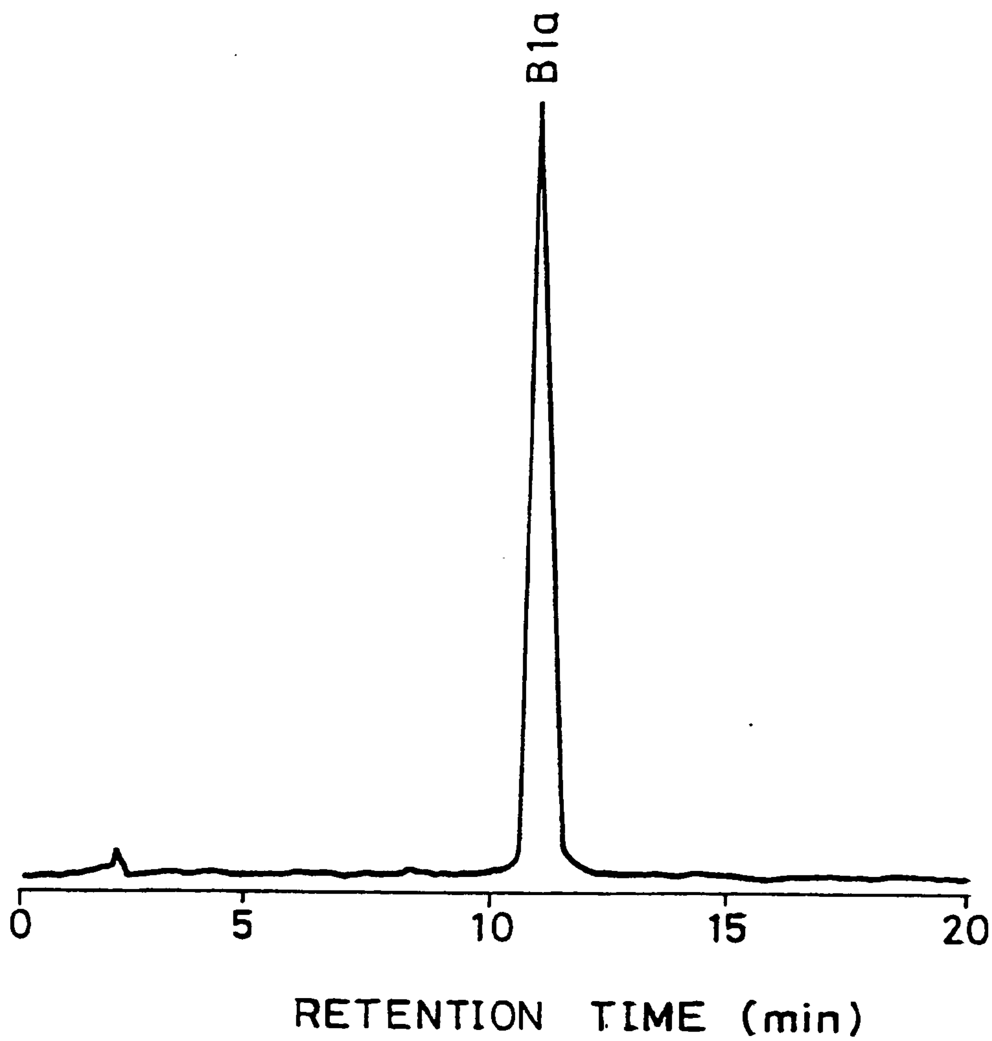


FIG. 3



European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 90308842.5

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	EP - A2 - 0 276 103 (PFIZER INC.) * Claim 5; pages 12-14 * --	1,2,8	C 12 N 1/20 C 12 P 17/18 C 12 P 1/06 //(C 12 N 1/20 C 12 R 1:465)
D,A	US - A - 4 310 519 (ALBERS-SCHONBERG et al.) * Abstract; claims * --	1-8	
A	EP - A2 - 0 313 297 (PFIZER INC.) * Abstract; claim 18 * ----	1,8	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

C 12 N
C 12 P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	24-01-1991	WOLF

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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